RESPONSE UNDER 37 C.F.R. 1.116
EXPEDITED PROCEDURE
EXAMINING GROUP 1644

S/N 10/663,158 PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	DeSauvage et al.	Examiner:	SKELDING, ZACHARY S
Serial No.:	10/663,158	Group Art Unit:	1644
Filed:	September 15, 2003	Docket No.:	11669.0123USC1
Conf. No.:	4053	Customer No.:	23552
Title:	TYPE I CYTOKINE RECEPTOR TCCR		

AMENDMENT UNDER 37 C.F.R. § 1.116

Mail Stop AF
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

Dear Sir:

In response to the Final Office Action mailed July 5, 2006, the date for timely reply extended three months from October 5, 2006 to January 5, 2007, please amend the above-identified application as follows:

Amendments to the Claims are reflected in the listing of claims that begins on page 2 of this paper.

Remarks begin on page 6 of this paper.

Amendments to the Claims:

This listing of claims will replace all prior versions and listings of claims in the application.

Listing of Claims:

1. (Withdrawn) A method of enhancing, stimulating or potentiating the differentiation of T-cells into the Th2 subtype instead of the Thl subtype, comprising contacting said T-cells with an effective amount of a TCCR antagonist.

2. (Withdrawn) The method of claim 1, wherein the enhancing, stimulating or occurs in a mammal and the effective amount is a therapeutically effective amount.

3. (Withdrawn) A method of treating a Thl-mediated disease in a mammal comprising administrating to said mammal a therapeutically effective amount of a TCCR polypeptide antagonist.

4. (Withdrawn) The method of claim 3, wherein the Thl-mediated disease is selected from the group consisting of autoimmune inflammatory disease and allograft rejection.

5. (Withdrawn) The method of claim 4, wherein the autoimmune inflammatory disease is selected from the group consisting of allergic encephalomyelitis, multiple sclerosis, insulin-dependent diabetes mellitus, autoimmune uveoretinitis, inflammatory bowel disease and autoimmune thyroid disease.

6. (Withdrawn) The method of claim 3, wherein the antagonist is a small molecule.

7. (Withdrawn) The method of claim 3, wherein the antagonist is an antisense oligonucleotide.

8. (Withdrawn) The method of claim 7, wherein the oligonucleotide is RNA.

9. (Withdrawn) The method of claim 7, wherein the oligonucleotide is DNA.

10. (Withdrawn) The method of claim 3, wherein the antagonist is a TCCR variant lacking biological activity.

11. (Withdrawn) The method of claim 3, wherein the antagonist is a monoclonal antibody.

12. (Withdrawn) The method of claim 11 wherein the antibody has nonhuman complementarity determining region (CDR) residues and human framework region (FR) residues.

13. (Withdrawn) The method of claim 3 wherein the antagonist is an antibody fragment or a single-chain antibody.

14. (Withdrawn) The method of claim 3 wherein the antagonist is a TCCR ligand.

15. (Currently Amended) A method of inhibiting or attenuating differentiation of ~~T-cells~~Th0 cells into a Th2 subtype, comprising administering to ~~undifferentiated T-cells~~ the Th0 cells an effective amount of a ~~TCCR polypeptide or~~, TCCR agonist antibody, or TCCR binding fragment thereof~~, wherein said polypeptide or agonist induces a TCCR-mediated response~~.

16. (Currently Amended) The method of claim 15, wherein the inhibiting or attenuating occurs in a mammal ~~and the effective amount is a therapeutically effective amount~~.

17. (Withdrawn) A method of treating a Th2-mediated disease in a mammal comprising ~~the~~ administration to said mammal a therapeutically effective amount of a TCCR polypeptide or agonist.

18. (Withdrawn) The method of claim 17, wherein the Th2-mediated disease is selected from the group consisting of: infectious diseases and allergic disorders.

19. (Withdrawn) The method of claim 18, wherein the infectious disease is selected from the group consisting of: *Leishmania major*, *Mycobacterium leprae*, *Candida albicans*, *Toxoplasma gonadi*, respiratory syncytial virus and human immunodeficiency virus

20. (Withdrawn) The method of claim 18, wherein allergic disorder is selected form the group consisting of: asthma, allergic rhinitis, atopic dermatitis and vernal conjunctivitis.

21. (Withdrawn) The method of claim 15, wherein the agonist is a small molecule.

22. (Withdrawn) The method of claim 15, wherein the agonist is a TCCR variant having biological activity.

23. (Previously presented) The method of claim 35, wherein the antibody is a monoclonal antibody.

24. (Previously presented) The method of claim 35, wherein the antibody is a humanized antibody.

25. (Previously presented) The method of claim 35, wherein the antibody fragment is a Fab, Fab', F(ab'), Fv, single-chain antibody, or a diabody.

26. (Withdrawn) The method of claim 15, wherein the agonist is a stable TCCR ECD.

27. (Withdrawn) A method for determining the presence of a TCCR polypeptide in a cell, comprising exposing the cell to an anti-TCCR antibody and measuring binding of the antibody to the cell, wherein binding of the antibody to the cell is indicative of the presence of TCCR polypeptide.

28. (Withdrawn) A method of diagnosing a Thl-mediated or Th2-mediated disease in a mammal, comprising detecting the level of expression of a gene encoding a TCCR polypeptide (a) in a test sample of tissue cells obtained from the mammal, and (b) in a control sample of known normal tissue cells of the same cell type, wherein a lower expression level in the test sample as compared to the control sample indicates the presence of a Th2-mediated disorder and a higher expression level in the test sample as compared to the control sample indicates the presence of a Th1-mediated disorder.

29. (Withdrawn) A method for identifying a compound capable of inhibiting the expression of a TCCR polypeptide comprising contacting a candidate compound with the polypeptide under conditions and for a time sufficient to allow these two components to interact.

30. (Withdrawn) The method of claim 29, wherein the candidate compound is immobilized on a solid support.

31. (Withdrawn) The method of claim 30, wherein the non-immobilized component carries a detectable label.

32. (Withdrawn) A method for identifying a compound capable of inhibiting a biological activity of a TCCR polypeptide comprising contacting a candidate compound with the polypeptide under conditions and for a time sufficient to allow these two component to interact.

33. (Withdrawn) The method of claim 32, wherein the candidate compound is immobilized on a solid support.

34. (Withdrawn) The method of claim 33, wherein the non-immobilized component carries a detectable label.

35. (Currently Amended) The method of claim 15, wherein said ~~agonist is an~~ antibody or a fragment thereof ~~that~~ binds SEQ ID NO: 1 or 2.

REMARKS

Claim Amendment

Claims 15, 16 and 35 have been amended to further clarify the claimed invention. Support for the amendment can be found in the specification, for example, at page 3, lines 8-9, page 10, lines 2-4, and Fig. 15. Applicants submit the amendment does not raise any issues of new matter and places the claims in condition for allowance.

After entry of the amendment, claims 1-35 will be pending. Claims 1-14, 17-22, and 26-34 have been withdrawn and claims 15, 16, 23-25, and 35 are under consideration by the Examiner.

Enablement

Claims 15, 16, 23-25, and 35 were rejected under 35 U.S.C. § 112, first paragraph, as lacking enablement. Applicants respectfully traverse this rejection.

Without acquiescing to the rejection and solely for the purpose of advancing prosecution, claim 15 has been amended to recite a method of inhibiting or attenuating differentiation of Th0 cells into a Th2 subtype, comprising administering to the Th0 cells an effective amount of a TCCR agonist antibody, or TCCR binding fragment thereof. Applicants reserve the right to pursue the canceled subject matter in a continuation application. Applicants submit claim 15 as amended fully complies with the enablement requirement.

The Office continues to allege the specification does not enable administering any TCCR agonist, including TCCR agonist antibodies. Applicants respectfully do not agree.

There are many factors to be considered in an analysis of enablement, including breadth of the claims, nature of the invention, the state of the prior art, the level of ordinary skill, level of predictability in the art, the amount of direction provided by the inventor and the existence of working examples, and the quantity of experimentation. MPEP 2164.01(a) citing *In Re Wands*, 858 F.2d 731, 737 (Fed. Cir. 1988). Only a reasonable correlation between the specification and the scope of enablement is required.

The claims as amended recite administering a TCCR agonist antibody or antigen binding to Th0 cells. As discussed in the previous response, the working examples demonstrate

inhibition of Th1 cytokine production and inhibition of Th1- mediated responses contrasted with exacerbated production of Th2 cytokines and exacerbated Th2 responses in TCCR-/- animals.

Further, the cytokines produced by Th1 cells and by Th2 cells each function to inhibit the other. As shown in the working examples, the lack of TCCR signaling results in the inability of differentiated T cells to produce Th1 cytokines, whereas production of Th2 cytokines is enhanced.

In the absence of TCCR receptor activation, an exacerbated Th2 response was induced. The exacerbated Th2 response was evidenced both by Th2-mediated immune response and by production of Th2 cytokines from T cells obtained from the TCCR-/- mice. These TCCR deficient mice and cells obtained from such mice exhibited a reduced Th1 response. Reduced Th1 cytokine production by T cells obtained from TCCR-/- mice, decreased total serum IgG2a, reduced titers or IgG2a in response to *in vivo* challenge with ovalbumin, and the inability to mount a Th1 response to bacterial infection fully demonstrate reduced development of Th1 cells in TCCR-/- mice. These data provide guidance about the role of TCCR receptor activation in mediating Th0 cell differentiation, and reasonably suggest that replacement of TCCR signaling with a TCCR agonist will inhibit or attenuate the exacerbated Th2 response and restore the diminished Th1 response. This guidance is consistent with the claimed methods.

Contrary to the Examiner's assertions, Lucas *et al.* and USSN 11/275,181 (hereinafter the '181 application) confirm Applicants' teachings. Lucas *et al.* discloses further studies into the mechanism of TCCR-mediated immune response, using the discovered TCCR ligand, IL27. The publication confirms a role for TCCR signaling in the suppression of Th2-mediated disease. Lucas *et al.* describes the effect of IL27/TCCR activation on "the master switch" for Th2 development, GATA3.

> Although IL-27 does not drive the differentiation process of $CD4^+$ cells toward an IFN-γ-secreting phenotype on its own, it certainly represents an important early trigger for TH1 differentiation *in vivo*, as demonstrated by the increased initial susceptibility of *TCCR*$^{/wsx-1}$ knockout mice to infection with intracellular pathogens (16, 17). Our *in vitro* studies show that IL-27, an early product of activated antigen-presenting cells (18), acts on naïve $CD4^+$ T cells by inducing IL-12 responsiveness by means of induction of T-bet and suppression of GATA-3. Therefore, at the time of activation of naïve $CD4^+$ T cell[s] by antigen-presenting cells *in vivo*, IL-27 functions in a paracrine manner to establish IL-12 responsiveness of early developing T_H cells, and consequently contributes to bias the T cell response toward a T_H1 outcome. (Lucas *et al.* at page 15052).

Lucas *et al.* also demonstrate that IL-27 provides an IFN-γ-independent signal for the induction of T-bet in developing T_H cells, and confirm that the TCCR agonist, IL-27, not only induces T-bet, leading to a T_H1 outcome, but "provides an alternative signal for GATA-3 suppression" thereby suppressing development of T_H2 cells and a T_H2 cell response (Lucas *et al.* at page 15052). Accordingly, Applicants assert the claimed methods are confirmed and supported by Lucas *et al.*

The Office Action alleges the '181 application does not provide a sufficient nexus between the functional activities of antibody 2686, a TCCR agonist antibody, and inhibiting or attenuating the differentiation of Th0 cells into the Th2 subtype because the Ba/F3 cells in Example 6 of the '181 application are not T cells. Applicants respectfully do not agree. The '181 application was cited for the purpose of confirming that TCCR agonist antibodies are capable of binding to TCCR expressed by a cell and inducing a TCCR-mediated biological activity. Proliferation of the Ba/F3 cells was mediated by binding of the TCCR agonist antibody to the recombinantly expressed human TCCR. Example 6 of the '181 application confirms that a TCCR agonist antibody, such as the antibody 2686, can bind and stimulate human TCCR expressed by a cell to induce a TCCR-mediated biological activity, such as cell differentiation or cell proliferation.

As discussed above, the claims as amended are directed to inhibiting or attenuating differentiation of Th0 cells into a Th2 subtype with an effective amount of a TCCR antibody, or TCCR binding fragment thereof. The specification teaches that TCCR signaling induces differentiation of Th0 cells into Th1 cells, and inhibits or attenuates differentiation of Th0 cells into Th2 cells. The specification further TCCR asserts signaling with a TCCR agonist such as an agonist antibody will inhibit or attenuate the exacerbated Th2 response and restore the diminished Th1 response.

These teachings are confirmed by subsequent publications. Pflanz *et al.*, 2002, *Immunity*, 16:779-790 (copy enclosed) confirms that a TCCR agonist, IL-27, induces naïve CD4+ T cells (Th0 cells) to differentiate into Th1 cells but not Th2 cells (Pflanz *et al.* at Figs. 5 and 6). Lucas *et al.*, as discussed above, confirms the TCCR agonist IL-27 provides an IFN-γ-independent signal for the inducing factor T-bet, leading to a T_H1 outcome, as well as inducing an alternative signal for GATA-3 suppression, thereby suppressing development of T_H2 cells and suppressing

T_H2 cell response (Lucas *et al.* at page 15052). The '181 application, as discussed above, confirms that a TCCR agonist antibody, such as antibody 2686, is capable of binding to TCCR expressed by a cell and the agonist binding stimulates/induces a TCCR-mediated biological activity (See, Example 6, '181 application) in the cell.

In view of the guidance and working examples provided in the specification and the confirmatory evidence provided by Pflanz *et al.*, Lucas *et al.*, and the '181 application, Applicants respectfully submit the specification provides sufficient evidence that administering a TCCR agonist such as a TCCR agonist antibody, would be reasonably expected to inhibit or attenuate differentiation of Th0 cells into the Th2 subtype. Based on the teachings of the specification, the full scope of the claims as amended can be practiced without undue experimentation. Withdrawal of the enablement rejection is respectfully requested.

Written Description

Claims 15, 16, 23-25, and 35 were rejected under 35 U.S.C. § 112, first paragraph, as lacking written description. Applicants respectfully traverse this rejection.

The Office Action alleges the specification fails to contain sufficient written description of any TCCR agonists. Without acquiescing to the rejection and solely for the purpose of advancing prosecution, claim 15 has been amended to recite a method of inhibiting or attenuating differentiation of Th0 cells into a Th2 subtype, comprising administering to the Th0 cells an effective amount of a TCCR agonist antibody, or TCCR binding fragment thereof. Applicants reserve the right to pursue the canceled subject matter in a continuation application.

To satisfy the written description requirement, a patent specification must describe the claimed invention in sufficient detail that one skilled in the art can reasonably conclude that the inventor had possession of the claimed invention. MPEP § 2163(I) (emphasis added). As discussed above for enablement, the specification provides adequate description of the structure of TCCR and its role in the development of T cells and T cell responses.

Applicants also direct the Examiner's attention to Example 16 in the Revised Written Description Guidelines Training Materials. Example 16 outlines a written description analysis of an antibody claim that satisfies the requirement under 35 U.S.C. § 112, first paragraph. The claim in Example 16 is directed to a genus of antibodies capable of binding antigen X. The

specification provided a clear protocol by which antigen X was isolated. Antigen X was purified by gel filtration and found to have a molecular weight of 55 KD. The specification did not disclose antibodies that specifically bind antigen X in an example. Example 16 in the written description guidelines states:

> Considering the routine art-recognized method of making antibodies to fully characterized antigens, the well defined structural characteristics for the five classes of antibody, the functional characteristics of antibody binding, and the fact that the antibody technology is well developed and mature, one of skill in the art would have recognized that the spectrum of antibodies which bind to antigen X were implicitly disclosed as a result of the isolation of antigen X.

Similar to the claim analyzed in Example 16 of the written description guidelines, Applicants' claims encompass a genus of antibodies that bind TCCR. Examples 1 and 4-7, in the specification describe TCCR nucleic acid and amino acid sequence, and how to isolate cDNA clones encoding TCCR and express TCCR in cells. Example 8 describes production of antibodies that specifically bind TCCR. The specification describes methods for identifying TCCR agonists, including TCCR agonist antibodies. See, for example, the specification at pages 63-65.

Applying the analysis set forth in Example 16 of the written description guidelines, Applicants submit the specification sufficiently describes the genus of antibodies. Considering the high and advanced level of skill in the art of antibody production, one skilled in the art would have recognized that the spectrum of antibodies that bind TCCR were implicitly disclosed as a result of the isolation of TCCR. The spectrum of antibodies that bind TCCR would include those agonist Ab that stimulate TCCR signaling and are useful in the claimed method invention.

For at least these reasons, Applicants respectfully submit the specification fully describes the claimed invention. Removal of the written description rejection is respectfully requested.

Provisional Double Patenting

Claims 15, 16, 23-25, and 35 were provisionally rejected under the judicially created doctrine of obviousness-type double patenting in view of U.S. Patent Application No.

10/088,950. Applicants acknowledge the rejection and respectfully request that the rejection be held in abeyance until notice of allowable subject matter.

New Matter

Claims 15, 16, 23-25, and 35 were rejected under 35 U.S.C. § 112, first paragraph, as failing to comply with the written description requirement. This is a new matter rejection. Applicants respectfully traverse the rejection.

The claims have been amended to clarify that the undifferentiated T cells are Th0 cells. Support for the amendment can be found in the specification, for example, at page 3, lines 8-9, page 10, lines 2-4, and Fig. 15. The phrase "induce a TCCR-mediated response" has been removed from the claims.

Withdrawal of the new matter rejection is respectfully requested.

Conclusion

In view of the above amendments and remarks, Applicants respectfully submit the claims are in condition for allowance and request a Notice of Allowance. If the Examiner believes a telephone conference would advance the prosecution of this application, the Examiner is invited to telephone the undersigned at the below-listed telephone number.

Respectfully submitted,

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Date: 21 December 2006

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IL-27, a Heterodimeric Cytokine Composed of EBI3 and p28 Protein, Induces Proliferation of Naive CD4$^+$ T Cells

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Summary

An efficient Th1-driven adaptive immune response requires activation of the T cell receptor and secretion of the T cell stimulatory cytokine IL-12 by activated antigen-presenting cells. IL-12 triggers Th1 polarization of naive CD4$^+$ T cells and secretion of IFN-γ. We describe a new heterodimeric cytokine termed IL-27 that consists of EBI3, an IL-12p40-related protein, and p28, a newly discovered IL-12p35-related polypeptide. IL-27 is an early product of activated antigen-presenting cells and drives rapid clonal expansion of naive but not memory CD4$^+$ T cells. It also strongly synergizes with IL-12 to trigger IFN-γ production of naive CD4$^+$ T cells. IL-27 mediates its biologic effects through the orphan cytokine receptor WSX-1/TCCR.

Introduction

Upon antigen exposure through contact with cells of the innate immune system, naive T cells undergo rapid clonal expansion and differentiation into several fundamental phenotypes characterized by the different effector proteins they secrete (Mosmann and Sad, 1996). To a large extent, this process is controlled by a complex network of soluble protein factors collectively termed cytokines. Of particular importance for this process are members of the four-helix bundle cytokines (Rozwarski et al., 1994). It is now well established that IL-4 predominantly directs the development of Th2 effector cells characterized by secretion of IL-4, IL-5, and IL-13 as an important step in fighting extracellular pathogens (Mosmann and Sad, 1996). IL-12 is a dominant factor in driving the development of Th1 cells leading to secretion of IFN-γ, which stimulates the immune response to eradicate intracellular pathogens. Other factors such as IL-2 are not associated with differentiation into a particular T cell phenotype but are thought to be sublineage independent T cell growth factors. More recent data have suggested a role for still additional cofactors in the development of committed Th1 and Th2 cells. IL-18, a member of the IL-1 family, does not drive Th1 development but augments IL-12-induced Th1 development and synergizes with IL-12 to induce the production of IFN-γ from these cells (Robinson et al., 1997). Several lines of evidence, however, suggest that still other factors exist with potentially critical contributions.

Recently, a novel member of the class I cytokine receptor family was identified, WSX-1 or TCCR (Chen et al., 2000; Sprecher et al., 1998), that showed structural homology to members of the IL-6/IL-12 subfamily of receptors, including IL-12Rβ1 and IL-12Rβ2, LIFR, OSMR, gp130, and G-CSFR. Mice deficient in TCCR showed a remarkably impaired Th1 response as measured by the production of IFN-γ when challenged in vivo with protein antigen and were found to be much more susceptible to infection with intracellular pathogens such as *Listeria monocytogenes* and *Leishmania major*. These data suggest that at least one additional as yet unidentified ligand exists that, together with IL-12 and IL-18, can act as a potent stimulator of Th1 responses.

Within the IL-6/IL-12 subgroup of class I cytokine receptors, Epstein-Barr virus-induced gene 3 (EBI3) represents a receptor with yet unknown function (Devergne et al., 1996). EBI3 lacks a membrane-anchoring motif and is predicted to be a secreted protein. Unlike WSX-1/TCCR, which is related to the signal transducing IL-6/IL-12 receptors, EBI3 is related to the specificity-determining receptors in this family, including IL-6Rα, IL-11Rα, CNTFRα, CLF-1, and in particular the shared p40 subunit of IL-12 and IL-23 (Oppmann et al., 2000). It has been reported that the p35 subunit of IL-12 can alternatively be complexed with EBI3 (Devergne et al., 1997), although so far no biologic function for this heterodimer has been demonstrated. Initially discovered in the supernatant of Epstein-Barr virus-transformed B cells, EBI3 is also expressed in vivo by activated antigen-presenting cells (APCs) and at very high levels by placental syncytiotrophoblasts.

In this report, we describe a novel family member of the long-chain four-helix bundle cytokines (Bazan, 1990). We show that this protein, designated p28, binds EBI3 to form a novel heterodimeric cytokine, which we have named IL-27. This novel heterodimeric factor is expressed by APCs in the early phase after antigen-mediated activation. IL-27 triggers rapid clonal expansion of antigen-specific naive human and mouse CD4$^+$ T cells. Moreover, it promotes Th1 polarization and IFN-γ production of naive CD4$^+$ T cells. These activities of IL-27 are dependent on simultaneous T cell receptor activation and occur in synergy with IL-12. Finally, we show binding of the p28/EBI3 heterodimer to the orphan cytokine receptor WSX-1/TCCR.

Results

p28 Was Identified Computationally

We searched sequence databases with a computationally derived profile (Gribskov et al., 1987) of members of the interleukin-6 (IL-6) helical cytokine family. This search led to the identification of a novel hematopoietic

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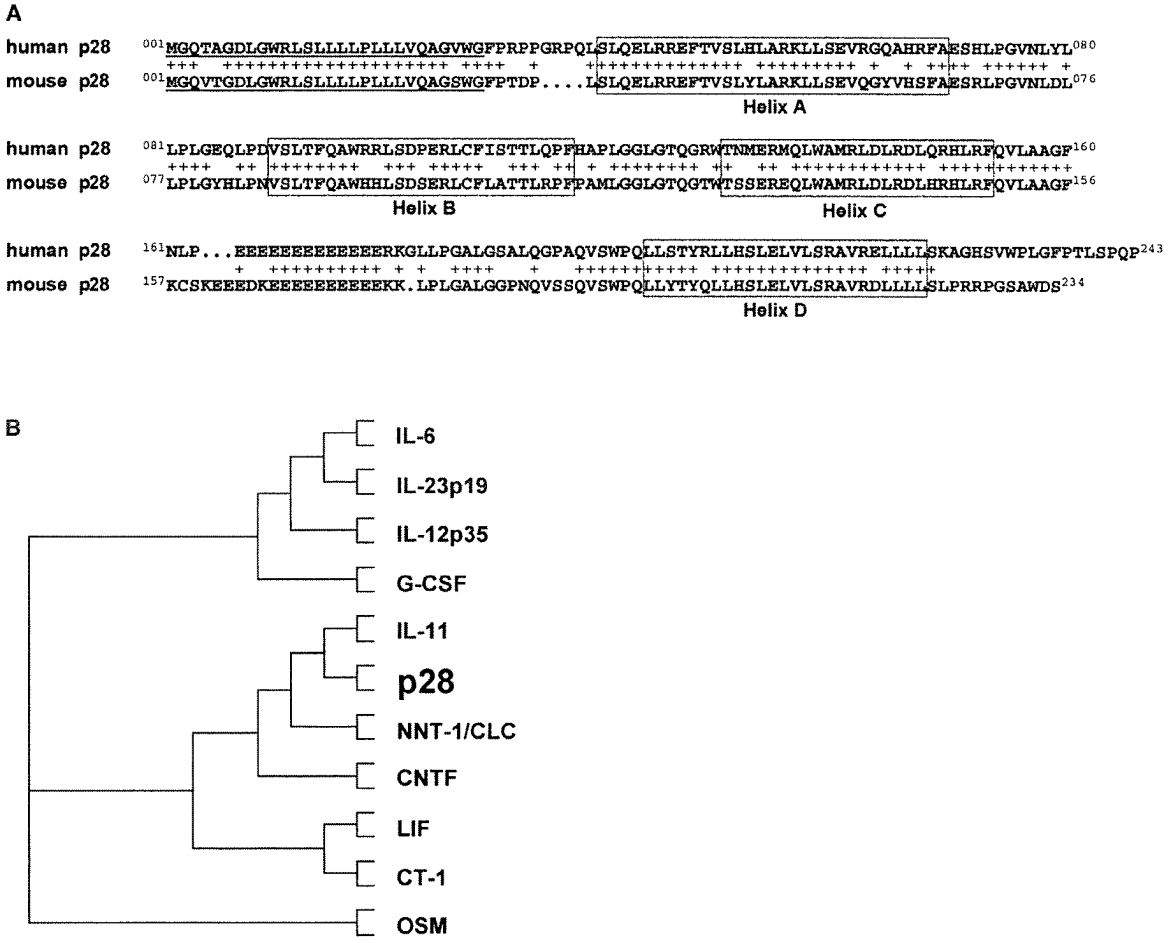


Figure 1. Alignment of Human and Mouse p28 Protein Sequences

(A) Sequence alignment of human and mouse p28 proteins. Boxes indicate the predicted four α helices A, B, C, and D (on the basis of secondary structure prediction and a ClustalX multiple sequence alignment). N-terminal amino acids constituting the predicted signal peptide are underlined.

(B) Evolutionary dendrogram for members of the family of long-chain four-helix bundle cytokines, human and mouse orthologs. The dendrogram is based on a ClustalX multiple sequence alignment. P28 is highlighted in bold characters.

cytokine (see Experimental Procedures for details). The cytokine was named p28 according to its apparent molar mass as determined by SDS-PAGE. The human p28 gene is located on chromosome 16p11. The p28 cDNA sequences encode 243/234 amino acid polypeptides (human and mouse, respectively; 1A) corresponding to mature proteins with calculated molar mass of 24.5 and 23.6 kDa. No N-glycosylation sites are found in hp28, but several O-glycosylation sites are predicted. Mouse p28 contains one potential N-glycosylation site (N85). Both human and mouse p28 display an unusual sequence insertion in the predicted loop region between helix C and D. In hp28 the C-D loop contains a stretch of 13 glutamic acid residues; mp28 displays 14 negatively charged residues in this region, interrupted by one lysine residue (Figure 1A). This highly charged sequence has not been observed in any other helical cytokine. Overall, human and mouse p28 are 73% identical. An evolutionary dendrogram displays p28 in the context of IL-6/IL-12 cytokine family members (Figure 1B).

p28 and EBI3 Form a Composite Factor

The presence of an N-terminal signal peptide suggested that p28 would be secreted as a soluble factor when expressed in mammalian cells. After HEK293T cells had been transiently transfected with C-terminal e-tagged (E) forms of mouse and human p28, only mp28-E (Figure 2A, lane 2) but not hp28-E protein (Figure 2B, top panel, lane 2) could be immunoprecipitated from the supernatants. However, hp28-E could be precipitated from the cellular lysates, indicating that the protein is expressed but not efficiently secreted (Figure 2B, bottom panel, lane 2). The three bands visible at apparent molar masses between 25 and 29 kDa most likely reflect different O-linked glycosylation states of hp28 in the cell lysates. Since mp28 was secreted, we tested either transfection supernatant or purified mp28 protein in various bioassays but were unable to detect biologic activity (data not shown). We next investigated the possibility that p28 is part of a heterodimeric factor like IL-12 (Gubler et al., 1991; Wolf et al., 1991) and requires coexpres-

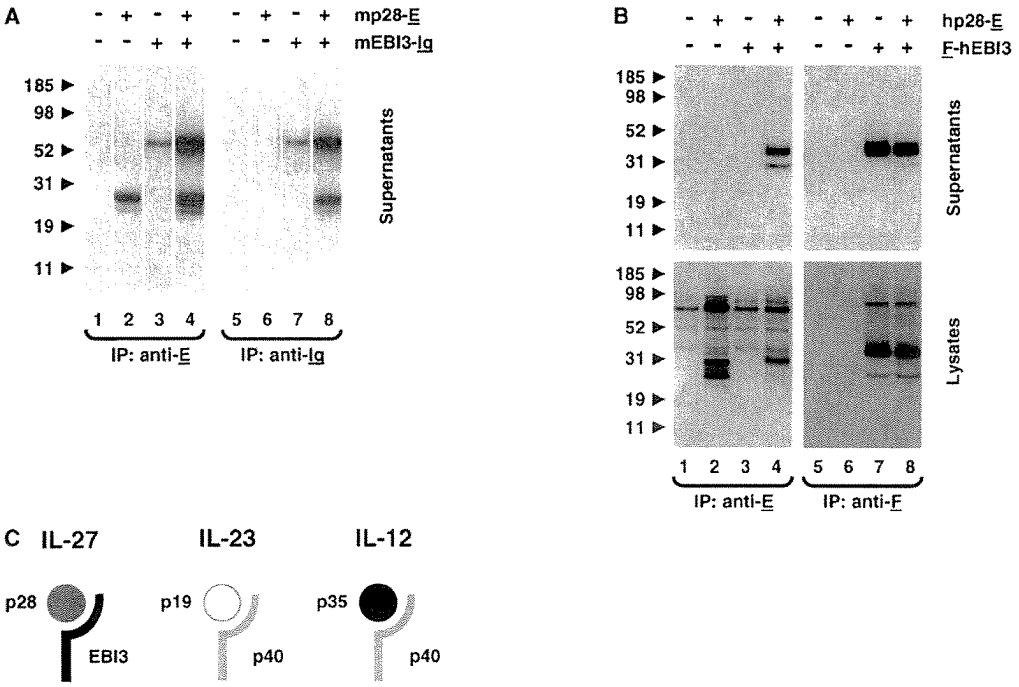


Figure 2. Coexpressed p28 and EBI3 Form a Complex Allowing Secretion of hp28

(A) HEK293T cells were transiently transfected with the following relative quantities of expression plasmids: control only (lanes 1 and 5), 50% mp28-E/50% control (lanes 2 and 6), 50% control/50% mEBI3-Ig (lanes 3 and 7), and 50% mp28-E/50% mEBI3-Ig (lanes 4 and 8).

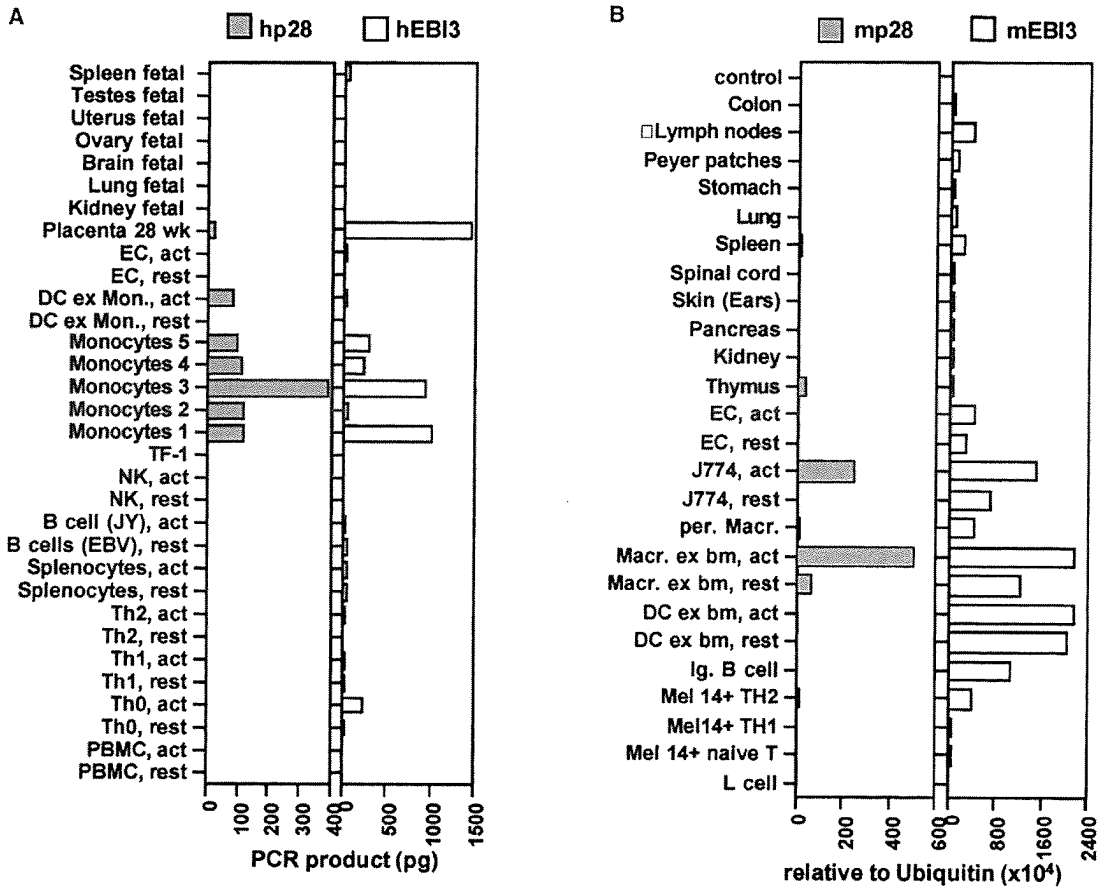
(B) HEK293T cells were transiently transfected with the following relative quantities of expression plasmids: control only (lanes 1 and 5), 50% hp28-E/50% control (lanes 2 and 6), 50% control/50% F-hEBI3 (lanes 3 and 7), and 50% hp28-E/50% F-hEBI3 (lanes 4 and 8). In both cases 24 hr after transfection, the cells were metabolically ^{35}S-labeled for 16 hr, and proteins were immunoprecipitated from cellular supernatants (A and B) or total cell lysates (B only) using protein G Sepharose-coupled anti-etag mAb (E) or flag-M2 agarose (F) or using protein G Sepharose (Ig), as indicated. Immunoprecipitated proteins were separated by SDS-PAGE and visualized by autoradiography. The electrophoretic mobility of molecular weight markers is indicated on the left of each panel.

(C) Schematic representation of the molecular composition of IL-27, IL-23, and IL-12.

sion of a partner protein for efficient secretion and bioactivity. Besides IL-12, two other heterodimeric factors have recently been described, IL-23 (Oppmann et al., 2000) and CLF-1/CLC (Elson et al., 2000). In all three cases, secretion of the helical cytokine subunit (IL-12p35, IL-23p19, and CLC) is dependent on coexpression of a soluble binding receptor (p40 for both p35 and p19 and CLF-1 for CLC), and only the composite factor possesses biologic activity. Hp28 was therefore coexpressed with various secreted nonsignaling receptors of the IL-6 family, including soluble IL-11 receptor, CLF-1, IL-12p40, and an N-terminal flag-tagged (F) form of EBI3 (Devergne et al., 1996). Among these molecules, only EBI3 coexpression permitted secretion of hp28 (Figure 2B, top panel, lane 4) while concomitantly reducing levels of hp28-E in the cell lysates (Figure 2B, bottom panel, lane 4). Moreover, hp28-E and F-hEBI3 were coimmunoprecipitated with antibodies against either protein tag (Figure 2B, top panel, lanes 4 and 8), indicating that p28 and EBI3 form a soluble complex. Because F-hEBI3 was secreted on its own and at much higher levels than hp28-E, only a light band of p28-E was detected when the immunoprecipitation occurred via EBI3 (Figure 2B, top panel, lane 8). When the complex was immunoprecipitated via p28, equivalent amounts of EBI3 and p28 were detected (Figure 2B, top panel, lane 4). As mentioned above, mp28 could be secreted independently of a partner protein. However, when coexpressed with a C-terminal Ig-tagged (Ig) version of mEBI3, cellular supernatants consistently contained increased levels of both secreted mp28-E and mEBI3-Ig (Figure 2A, lanes 4 and 8). Similar to their human orthologs, mp28 and mEBI3 could be coimmunoprecipitated as a heterodimeric complex (Figure 2A, lane 8). A small amount of mp28 migrated at a slightly lower molecular weight. The lower band most likely represents unglycosylated mp28. Note that mEBI3-Ig precipitation in the anti-etag pull-down (Figure 2A, lane 3) is not due to nonspecific binding of the antibody but results from the fact that the anti-etag mAb is coupled to protein G beads. Protein G per se has a good affinity for the constant portion of various immunoglobulins. Figure 2C shows the molecular composition of IL-27 as compared to related heterodimeric family members IL-12 and IL-23.

Coexpression of p28 and EBI3 in Antigen-Presenting Cells

Since secretion of hp28 requires the presence of EBI3, we have investigated where both proteins are coexpressed. Analysis of a large panel of human and mouse cDNA libraries by real-time quantitative PCR showed


A
hp28
hEBI3
Spleen fetal
Testes fetal
Uterus fetal
Ovary fetal
Brain fetal
Lung fetal
Kidney fetal
Placenta 28 wk
EC, act
EC, rest
DC ex Mon., act
DC ex Mon., rest
Monocytes 5
Monocytes 4
Monocytes 3
Monocytes 2
Monocytes 1
TF-1
NK, act
NK, rest
B cell (JY), act
B cells (EBV), rest
Splenocytes, act
Splenocytes, rest
Th2, act
Th2, rest
Th1, act
Th1, rest
Th0, act
Th0, rest
PBMC, act
PBMC, rest
0 100 200 300 400 500 1000 1500
PCR product (pg)
B
mp28
mEBI3
control
Colon
Lymph nodes
Peyer patches
Stomach
Lung
Spleen
Spinal cord
Skin (Ears)
Pancreas
Kidney
Thymus
EC, act
EC, rest
J774, act
J774, rest
per. Macr.
Macr. ex bm, act
Macr. ex bm, rest
DC ex bm, act
DC ex bm, rest
Ig. B cell
Mel 14+ TH2
Mel14+ TH1
Mel 14+ naive T
L cell
0 200 400 600 800 1600 2400
relative to Ubiquitin (x10⁴)
C
p28
IL-12p35
EBI3
IL-12p40
D
A (405 nm)
protein (ng/ml)
0.01 0.1 1 10 100
E
p = 0.0002
A (405 nm)
medium DCs + CD40L DCs + CD40L/IFNg/LPS

that expression of p28 and EBI3 is highly restricted. Both mRNAs are primarily found in cells of myeloid lineage. Highest levels of human mRNAs were found in LPS-activated monocytes and monocyte-derived dendritic cells (DCs) (Figure 3A). A very high level of hEBI3 mRNA but not hp28 was seen in placenta. This observation is in agreement with earlier reports of high levels of EBI3 protein in placental syncytiotrophoblasts (Devergne et al., 1997). A similar pattern emerged when we analyzed the expression profile of mouse p28 and EBI3. Highest levels of both mp28 and mEBI3 was in activated macrophages (Figure 3B). Since antigen-presenting cells are also the primary source of IL-12, we studied the kinetics of production of IL-12p35, IL-12p40, p28, and EBI3 by monocyte-derived DCs stimulated with LPS. Human monocytes, isolated from peripheral blood and stimulated with GM-CSF and IL-4 for 6 days, were activated by LPS for various time intervals, and mRNA levels of IL-12p35, IL-12p40, p28, and EBI3 were analyzed by real-time quantitative PCR (Figure 3C). Despite donor to donor variations in the absolute amounts of PCR product, the kinetics recorded were consistent and revealed subtle differences among the four investigated proteins. After a short initial lag phase, message levels for IL-12p35 and IL-12p40 rapidly increased and consistently peaked between 8 and 14 hr of LPS stimulation and then dropped back to background level after 24 hr. A transient expression was also observed for p28, although maximal message levels were already found after 3–6 hr. Similar to IL-12, mRNA levels for p28 declined to background levels after 24 hr. In contrast, EBI3 showed less transient expression although its transcription was induced as early as 3 hr after LPS stimulation. Reaching maximal EBI3 mRNA levels between 12 and 24 hr, EBI3 message was still above nonstimulated background levels after 72 hr. The variation in p28/EBI3 levels between donors is much larger than that observed for p35/p40, suggesting that regulation of IL-27 possibly is less stringent than that of IL-12.

In order to detect IL-27 protein in the supernatant of activated DCs, a sandwich-ELISA was set up with a pair of anti-hIL-27 mAbs obtained from immunization of rats with the recombinant protein. This pair of anti-hIL-27 mAbs detects hIL-27 to a concentration of 600 pg/ml but does not detect free hEBI3 (Figure 3D). Using this sandwich-ELISA, hIL-27 protein was detected in the supernatants of CD40L or CD40L/LPS/IFN-γ activated DCs. After 36 hr of stimulation, the supernatants were harvested from the cells, concentrated 4-fold, and subjected to the ELISA assay. We calculated the concentration of IL-27 to be about 0.5 ng/ml in the supernatants of CD40L/LPS/IFN-γ activated DCs with a lower amount if cells were only stimulated with CD40L (Figure 3E).

IL-27 Specifically Acts on Naive Mouse and Human T Cells

To investigate the biologic effects of the IL-27 heterodimer, we engineered both human and mouse soluble tagged fusion proteins by flexibly linking the EBI3 chain to the p28 polypeptide. This methodology permits convenient expression and purification of a functional one-chain cytokine (Fischer et al., 1997). Similarly, IL-12 and IL-23 fusion proteins show specific activities identical to the native heterodimeric complexes (Anderson et al., 1997; Oppmann et al., 2000). We investigated the role of IL-27 on both mouse and human CD4+ T cell subsets. Sorted human naive T cells (CD4+CD45RA) and memory T cells (CD4+CD45RO) were stimulated with anti-CD3 mAb/anti-CD28 mAb for 4 days in the presence of anti-IL-2 mAb, and hIL-27 was titrated into the cultures. [^{3}H]TdR incorporation was measured to assess proliferation induced by IL-27. Human naive but not memory T cells proliferate dose dependently in response to IL-27 (Figure 4A). In a similar experiment, mouse naive T cells (CD4+CD45RB^{high}) and memory/activated T cells (CD4+CD45RB^{low}) were stimulated with anti-CD3 mAb/anti-CD28 mAb for 4 days in the presence of anti-IL-2 mAb, and mIL-27 was titrated into the cultures. Again, IL-27 triggered proliferation of naive but not memory T cells in a dose-dependent manner (Figure 4B). In the presence of anti-CD28 mAb, IL-12 did not enhance proliferation in response to IL-27 any further. However, in the absence of costimulation via anti-CD28, IL-27-driven proliferation of naive T cells was profoundly enhanced by IL-12, revealing synergy between IL-27 and IL-12 (data not shown). Thus, IL-27-dependent proliferation can be enhanced by costimulatory signals through either CD28 or the IL-12 receptors. IL-27-induced proliferation is dependent on simultaneous crosslinking of CD3/TCR, since no proliferation was observed in the absence

Figure 3. Distribution of p28 and EBI3 in Tissues and Cell Types and Detection of hIL-27 Protein

(A and B) Real-time quantitative PCR was performed for p28 and EBI3 on various human (A) and mouse (B) cDNA libraries. Human cDNA libraries index (top to bottom): various fetal tissues; term placenta week 28; endothelial cells (EC) activated and resting; monocyte-derived dendritic cells (DC) activated with LPS and resting; monocytes activated with LPS and/or IFN-γ for various times and in the presence and absence of IL-10 or anti-IL-10; hematopoietic precursor TF1; natural killer cells activated and resting; B cell lines activated and resting; splenocytes activated and resting; T cell subsets activated and resting; peripheral blood mononuclear cells activated and resting. Mouse cDNA libraries index (top to bottom): various tissues; endothelial cells activated and resting; J774 macrophage cell line activated with LPS and resting; peritoneal macrophages; macrophages from bone marrow activated with LPS and resting; dendritic cells from bone marrow activated and resting; B cells; Mel14+ T cell subsets; L-fibroblasts.

(C) Quantitative PCR analysis of p28, EBI3, IL-12p35, and IL-12p40 mRNA levels from activated human dendritic cells. Monocyte-derived dendritic cells from two donors were stimulated with LPS for various time intervals. RNA was isolated and the samples were analyzed by real-time quantitative PCR (Donor 1, squares/dashed line; Donor 2, triangles/solid line).

(D) Standard curves recorded for purified recombinant hIL-27 (black circles) and hEBI3 (gray squares) by sandwich-ELISA. Every point is the means of a triplicate experiment with standard errors. The means of a triplicate for a background control in the absence of recombinant protein is also shown (black diamond).

(E) Monocyte-derived dendritic cells were obtained and cultured as described in Experimental Procedures; after 36 hr supernatants were harvested, concentrated 4-fold, and put into the sandwich-ELISA. Every bar is the means of a triplicate experiment with standard errors. Statistical significance of the signal measured in column two versus three was calculated by applying a two-tailed Student's t test.

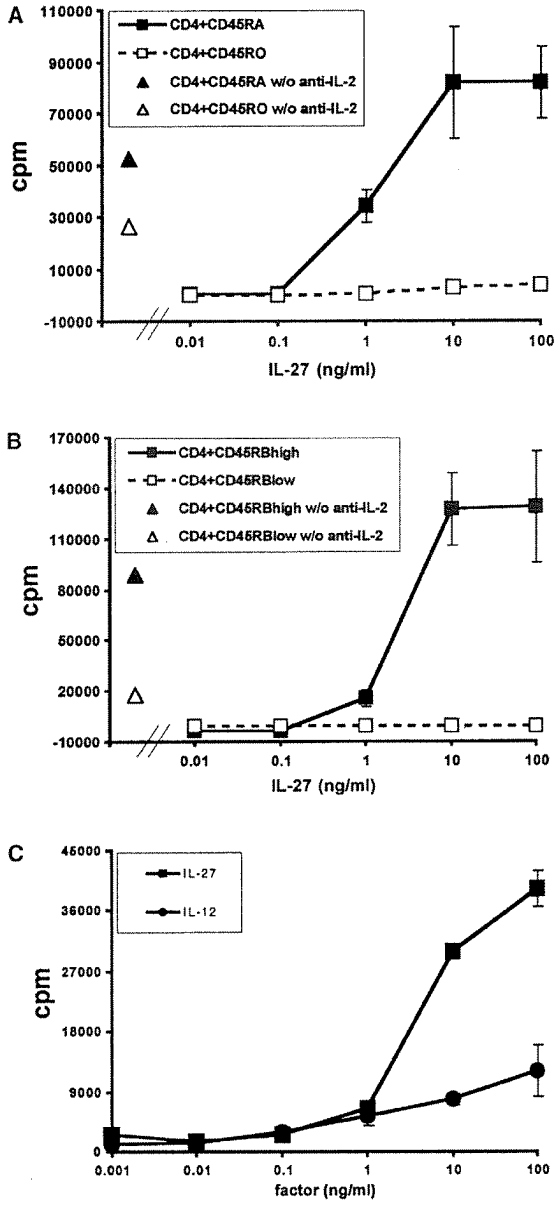


Figure 4. IL-27 Induces Proliferation of Naive but Not Memory $CD4^+$ T Cells

(A) Sorted human $CD4^+CD45RA$ (naive) and $CD4^+CD45RO$ (memory) T cells were cultured with plate-bound anti-CD3 mAb and soluble anti-CD28 mAb, anti-IL-2, and anti-IL-2R mAbs in the absence or presence of IL-27. After 5 days, [^{3}H]TdR incorporation (cpm) was assessed to measure factor-dependent proliferation. Proliferative responses to anti-CD3 mAbs and anti-CD28 mAbs only are also indicated.

(B) Sorted mouse $CD4^+CD45RB^{high}$ (naive) and $CD4^+CD45RB^{low}$ (memory) T cells were cultured with plate-bound anti-CD3 mAb and in the presence of anti-CD28 mAb and a neutralizing anti-IL-2 mAb. IL-27 was titrated into the cultures; control cells were cultured without IL-27. After 5 days, [^{3}H]TdR incorporation (counts per minute) was assessed to measure factor-dependent proliferation. Proliferative responses to anti-CD3 mAbs and anti-CD28 mAbs only are also indicated.

(C) Sorted mouse $CD4^+CD45RB^{high}$ T cells were cultured with plate-bound anti-CD3 mAb and in the presence of a neutralizing anti-IL-2 mAb for 72 hr. Then, cells were stimulated with titrated amounts of IL-27 or IL-12 for 24 hr, and [^{3}H]TdR incorporation (counts per minute) was assessed to measure factor-dependent proliferation. In all cases, data shown are corrected for background [^{3}H]TdR incorporation obtained in the absence of growth factor and are the mean of three experiments with standard deviations as indicated.

of CD3 activation. The same maximal proliferative response could be induced by stimulation with conditioned medium of p28/EBI3 cotransfected cells (data not shown). To compare the abilities of IL-27 and IL-12 to induce proliferation of naive $CD4^+$ T cells, FACS-sorted mouse $CD4^+CD45RB^{high}$ T cells were precultured with plate-bound anti-CD3 mAb, and either IL-27 or IL-12 was titrated into the cultures. IL-27 proved to be a much more potent proliferative stimulus for these cells (Figure 4C).

IL-27 Synergizes with IL-12 to Produce IFN-γ in Human and Mouse Naive T Cells

We measured the ability of human and mouse IL-27 to induce the production of IFN-γ in the presence of a neutralizing anti-IL-2 mAb, with costimulation via anti-CD3 or anti-CD3/anti-CD28 and both in the absence and presence of IL-12. In this assay, neither hIL-27 nor hIL-12 by itself induced IFN-γ production in anti-CD3- or anti-CD3/anti-CD28-activated $CD4^+CD45RA$ T cells. IFN-γ production was only observed in the presence of both cytokines, indicating strong synergy between IL-27 and IL-12 (Figure 5A). We have tested the possibility that the observed increase in IFN-γ levels reflects a downregulation of the IFN-γRs and thus decreased cytokine uptake rather than increased cytokine production. However, quantitative rt-PCR analysis showed that mRNA levels for IFN-γR1 and IFN-γR2 remained unchanged after IL-12 or IL-27 stimulation (data not shown). Sorted mouse $CD4^+CD45RB^{high}$ naive T cells were stimulated for 4 days with anti-CD3 mAb alone or with anti-CD3 mAb/anti-CD28 mAb and saturating amounts of IL-27 and IL-12. In the absence of anti-CD28 costimulation, neither IL-27 nor IL-12 by itself was capable of inducing substantial amounts of IFN-γ (Figure 5B). However, the combination of IL-27 and IL-12 induced up to about 300 ng/ml of IFN-γ. With anti-CD3/anti-CD28 costimulation, IL-27 as well as IL-12 was capable of inducing IFN-γ production. The combination of both factors led to an additive effect, with IFN-γ levels up to 550 ng/ml.

IL-27 Enhances IFN-γ Production in Human NK Cells

The synergistic activity of IL-27 and IL-12 on the production of IFN-γ by activated naive T cells led us to investigate the effects of IL-27 on NK cells. Highly purified human NK cells were isolated from PBMC and cultured in the presence of IL-2, IL-12, and IL-2 plus IL-12 in the absence or presence of IL-27. Low levels of IFN-γ were produced by NK cells cultured in the presence of IL-2 or IL-12, and these were not affected by addition of IL-27 (Figure 5C). However, IL-27 significantly increased the production of IFN-γ when NK cells were cultured in the presence of IL-2 and IL-12. Depending on the donor,

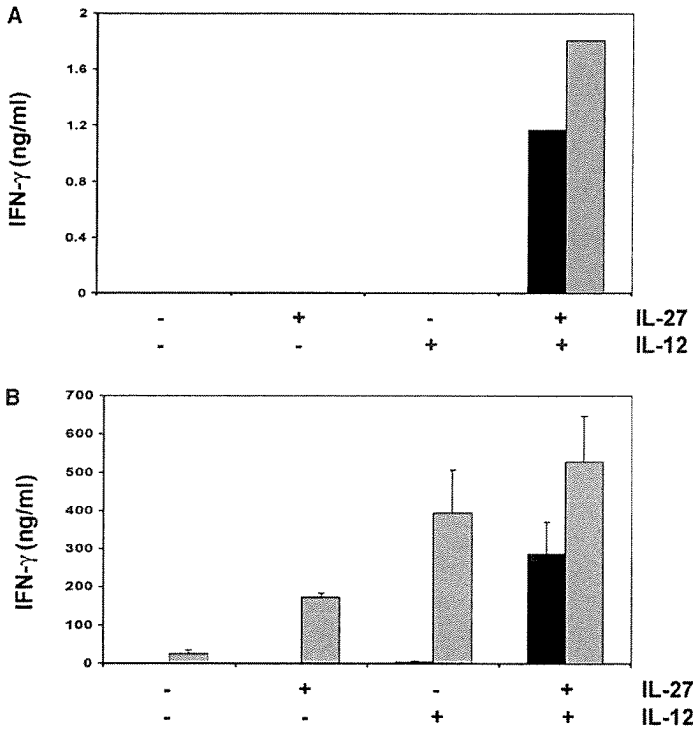


Figure 5. IL-27 Synergizes with IL-12 to Induce Th1 Polarization of Naive CD4+ T Cells

(A) Sorted human CD4+CD45RA T cells cultured with plate-bound anti-CD3 mAb and a neutralizing anti-IL-2 mAb. IL-27 and IL-12 were added as indicated in the absence (black bars) or presence (gray bars) of anti-CD28 mAb. IFN-γ production induced by the respective factors was quantified by ELISA. A representative out of three experiments is shown.

(B) Sorted mouse CD4+CD45RB^{high} T cells were cultured with plate-bound anti-CD3 mAb and a neutralizing anti-IL-2 mAb. IL-27 and IL-12 were added as indicated in the absence (black bars) or presence (gray bars) of anti-CD28 mAb. IFN-γ production induced by the respective factors was quantified by ELISA. Data shown represent mean and standard deviation of two independent experiments.

(C) IL-27 enhances IFN-γ production by NK cells cultured with IL-2 and IL-12. FACS-purified NK Cells were cultured in medium, IL-2, IL-12, or IL-2 and IL-12 in the absence or presence of IL-27. Culture supematants were harvested after 72 hr, and IFN-γ production was determined by ELISA (the given values are concentrations of IFN-γ in ng/ml). Data shown are from four different donors.

C

	medium	IL-27	IL-2	IL-12	IL-27 IL-2	IL-27 IL-12	IL-2 IL-12	IL-27 IL-2 IL-12
Donor 1	0	0	0.4	0.4	0.5	1	16	59
Donor 2	0	0	0.4	1.5	0.2	1	3.8	9.8
Donor 3	0	0	9.6	12.2	5.1	7.4	4.2	42
Donor 4	0	0	0.2	0	0.5	5.5	2.9	24

IL-27 increased the IFN-γ production 4- to 10-fold (Figure 5C). These results indicate that both activated (naive) T cells and NK cells are responsive to IL-27 and enhance IL-12-mediated IFN-γ production.

IL-27 Does Not Support Th2 Cytokine Production by Activated T Cells

Sorted mouse CD4+CD45RB^{high} T cells were cultured with plate-bound anti-CD3 and anti-CD28 in the presence of IL-4 and IL-27. Including IL-27 in the cultures led to a decreased IL-13 production both in the absence and presence of IL-4 (Figure 6). Thus, while inducing a strong Th1 response, IL-27 does not appear to promote Th2 cytokine production.

IL-27 Binds to WSX-1/TCCR

Because of the relationship between IL-27 and the IL-6/IL-12 family, we have concentrated our search for the IL-27 signaling receptors on this family. Members of this family were introduced into Ba/F3 cells and tested for binding to p28/EBI3. Of the receptors tested, only Ba/F3 cells expressing the orphan cytokine receptor WSX-1/TCCR (Chen et al., 2000; Sprecher et al., 1998) showed binding to tagged p28/EBI3. Ba/F3 cells infected with retroviral constructs expressing either F-tagged human or mouse WSX-1 (F-hWSX-1 or F-mWSX-1) showed cellular staining using anti-flag mAb (Figure 7A). Cells expressing F-hWSX-1 were then incubated with either hEBI3-Ig alone or with coexpressed hp28-E and EBI3-Ig. Heterodimeric p28/EBI3 bound to WSX-1 (Figure 7B); EBI3-Ig itself showed no detectable binding. Similarly, only the combination of mp28-E and mEBI3-Ig provided a detectable interaction with mWSX-1-expressing Ba/F3 cells, whereas the two individual proteins were not able to do so (Figure 7C). Incubation of independently expressed mp28-E and mEBI3-Ig with F-mWSX-1-expressing Ba/F3 cells also led to cellular staining (data not shown). Untransfected control cells were not stained by p28/EBI3. These results were confirmed by coimmunoprecipitation experiments using a soluble extracellular form of hWSX-1 with a C-terminal RSGH$_6$-tag (R). Only when all three proteins were present (hp28-E, F-hEBI3, and shWSX-1-R), immunoprecipitation of one protein brought down both other components independently of the immunoprecipitating antibody used (Figure 7D). The same coimmunoprecipitation experiment using the respective mouse orthologs had similar results (data not shown). To address the question of whether WSX-1

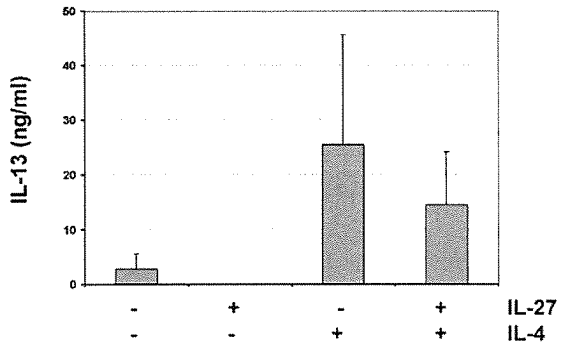


Figure 6. IL-27 Does Not Support Th2 Cytokine Production in Naive $CD4^+$ T Cells

Sorted mouse $CD4^+CD45RB^{high}$ T cells were cultured with plate-bound anti-CD3 mAb, anti-CD28 mAb, IL-27, and/or IL-4 as indicated. IL-13 expression induced by the respective factors was quantified by ELISA. Data shown represent mean and standard deviations of three independent experiments.

was sufficient to mediate IL-27 signal transduction, we tested proliferation of Ba/F3 cells expressing human or mouse WSX-1. These cells proliferate in response to IL-3 but did not proliferate in response to IL-27 (data not shown). Thus, WSX-1 appears to be required but not sufficient for IL-27-mediated signal transduction. Since receptors for IL-12, IL-23, and IL-27 are all related, it is likely that an additional IL-27R component resembles members of this family. Experiments using IL-12Rβ1 and/or IL-12Rβ2, in combination with WSX-1 in Ba/F3 cells, have indicated that these proteins are not part of the IL-27 receptor complex (data not shown). The identification of additional IL-27 signal-transducing receptor subunits is currently in progress.

Discussion

In this study, we describe the discovery and the initial biological characterization of IL-27, a heterodimeric cytokine composed of a newly identified helical subunit termed p28 and the soluble cytokine receptor EBI3. Expression of IL-27 appears restricted to antigen-presenting cells. This led us to suspect T cells of being a potential target of IL-27. We report here that human and mouse IL-27 potently induces the proliferation of naive T cells ($CD4^+CD45RA$ and $CD4^+CD45Rb^{high}$, respectively) but not memory T cells. The proliferative response is dependent on TCR/CD3 activation and can be enhanced by costimulatory signals, either CD28 or IL-12. IL-27 also synergizes with IL-12 to trigger IFN-γ production of naive $CD4^+$ T cells, driving these cells to a Th1 phenotype. These biologic effects are likely mediated via the receptor WSX-1/TCCR, although additional receptor components appear to be necessary.

IL-27 is in many aspects closely tied to two other cytokines, IL-12 and IL-23. All three cytokines are heterodimers that consist of related helical subunits (IL-12p35, IL-23p19, and IL-27p28) and related soluble cytokine receptor subunits (IL-12p40, IL-23p40, and IL-27EBI3). In addition to the structural similarity, the p28 subunit of IL-27 either is not (human p28) or is inefficiently (mouse p28) secreted on its own, as has been described for IL-12p35 and IL-23p19 (Oppmann et al., 2000; Trinchieri, 1995). Instead, secretion of p28 is dependent on simultaneous expression of the soluble accessory receptor EBI3, functioning as cofactor to permit secretion of the helical cytokine subunit as part of the heterodimeric IL-27 complex. Quantitative PCR analysis revealed that coexpression of p28 and EBI3 is restricted to APCs. These cells are also the main source of IL-12 and IL-23 production, suggesting that IL-27 may have similar T cell targets. In fact, IL-27 potently induced a proliferative response on naive $CD4^+$ T cells but had no effect on $CD4^+$ memory T cells. IL-27 also strongly synergized with IL-12 to produce very high levels of IFN-γ by activated naive T cells, setting the stage for commitment to a Th1 phenotype. When combined with IL-4, IL-27, like IL-12 and IL-23, was unable to enhance Th2 polarization. The interrelationship between IL-27, IL-12, and IL-23 extends to the cellular receptors for these molecules. WSX-1/TCCR, a thus far orphan receptor, was identified as a component of the IL-27 receptor. This receptor is most closely related to the IL-12 receptors, and its gene is located on the same chromosome as IL-12Rβ1 (human chromosome 19).

The p28 subunit of IL-27 aligns well with the IL-6/IL-12 family of long-chain four-helix bundle cytokines with the exception of a unique stretch of 13 glutamic acid residues in both human and mouse p28, interrupted by a DK-dipeptide in mouse p28. A similarly prominent insertion of charged residues has not been observed in other cytokines. Its location in the proposed loop between helix C and D is unlikely to interfere with the overall helical fold of the protein or with the interaction of the protein with its cellular receptor(s), which for this family primarily takes place via residues in the A and D helices (Bazan, 1990).

The discovery of IL-27 with an apparent biologic profile similar to that of IL-12 raises the question of what possibly differentiates these two factors. IL-12 is a dominant factor produced early on by phagocytic cells in response to bacteria and intracellular parasites (Gately et al., 1998; Trinchieri, 1998). Once produced in response to infection, IL-12 is thought to act first on NK cells and T cells to induce the production of IFN-γ. This sets off a cascade of events ultimately leading to optimal IFN-γ production and to proliferation of fully differentiated Th1 cells in response to antigen (Trinchieri, 1998). The early and potent responses we observed with IL-27 on naive $CD4^+$ T cells led us to investigate the kinetics of LPS-induced expression of p28, EBI3, and the two IL-12 subunits by APCs to possibly obtain some insight into the physiological roles of these molecules. Consistent with previous reports (Hashimoto et al., 2000), expression kinetics for IL-12p35 and IL-12p40 are transient and superimposable; both subunits show LPS-inducible expression with highest levels between 8 and 14 hr. After 24 hr, expression has almost declined to background levels. The p28 gene is also transiently induced by LPS but appears to be upregulated more quickly than IL-12. With EBI3 being rapidly upregulated as well, IL-27 appears to be present at an earlier time after APC stimulation. Thus, it is conceivable that the physiological role of p28/EBI3 precedes the actions of IL-12. The extended presence of EBI3 mRNA might point to additional p28-

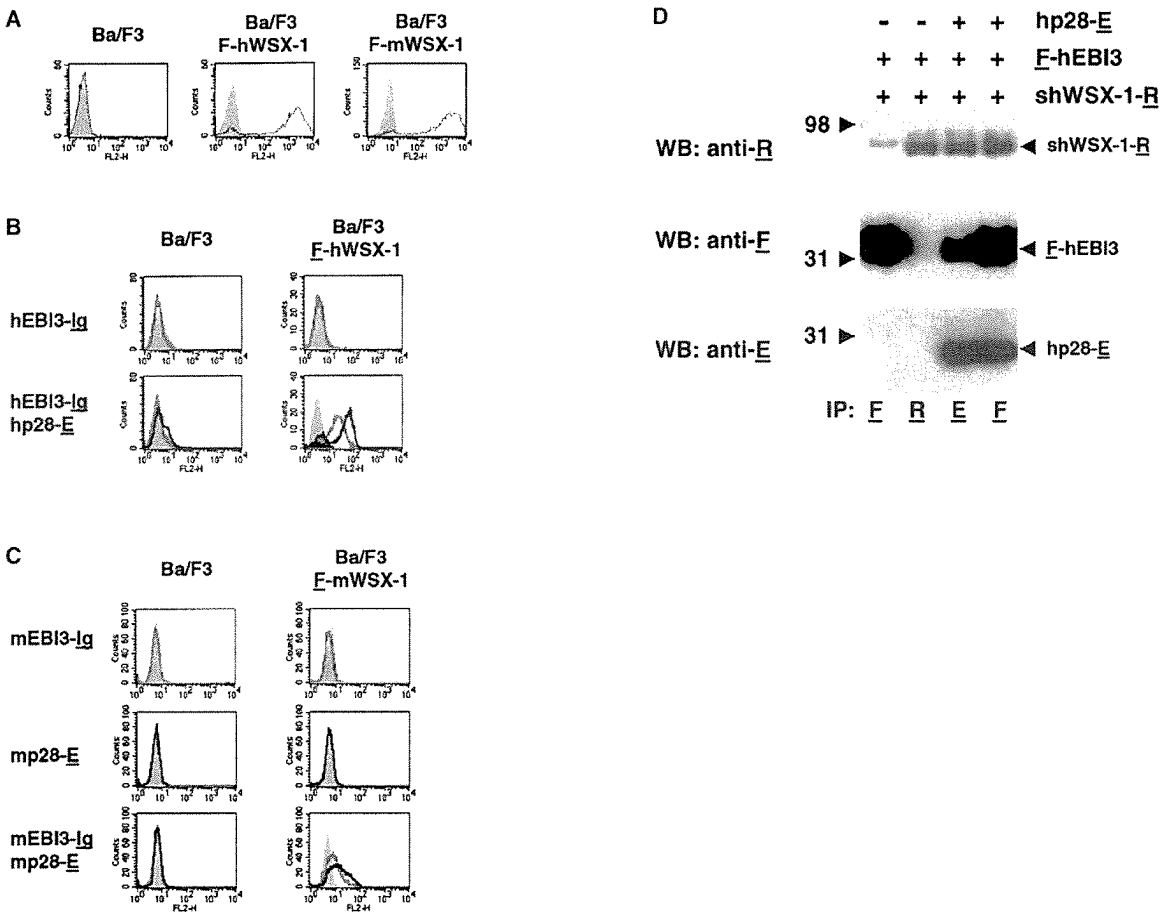


Figure 7. The p28/EBI3 Complex Binds to WSX-1

Ba/F3 cells were stably transfected with cDNAs encoding either F-hWSX-1 or F-mWSX-1 or left untransfected.

(A) Cell surface expression of F-hWSX-1 and F-mWSX-1. Light gray peaks represent cell-associated fluorescence after isotype control mAb + PE; open peaks with the black dotted line represent F + PE.

(B) Untransfected Ba/F3 cells or Ba/F3 cells expressing F-hWSX-1 were incubated with the supernatants of transiently transfected HEK293T cells containing hEBI3-Ig or coexpressed hp28-E/hEBI3-Ig proteins, as indicated. Light gray peaks represent cell-associated fluorescence after isotype control mAb + PE; open peaks with the dark gray line represent Ig + PE; open peaks with the black line represent E + PE.

(C) Untransfected Ba/F3 cells or Ba/F3 cells expressing F-mWSX-1 were incubated with the supernatants of transiently transfected HEK293T cells containing mEBI3-Ig, mp28-E, or coexpressed mp28-E/mEBI3-Ig proteins, as indicated. Light gray peaks represent cell-associated fluorescence after isotype control mAb + PE; open peaks with the dark gray line represent Ig + PE; open peaks with the black line represent E + PE.

(D) Coimmunoprecipitation of the temary complex hp28/hEBI3/shWSX-1. Supernatants of transiently transfected HEK293T cells containing either F-hEBI3 or coexpressed hp28-E/F-hEBI3 were combined with supernatants containing shWSX-1-R as indicated, and immunoprecipitations were performed using mAbs against the three different epitope tags. Precipitated proteins were separated by SDS-PAGE, electroblotted, and visualized by Western blot/ECL.

independent functions of this protein at the later stages of an immune response.

We identified the orphan WSX-1/TCCR as a subunit of the IL-27 receptor. It is expressed in adult lymphoid tissues including PBLs, thymus, and spleen. Within the lymphoid compartment, highest expression has been observed on CD4+ T cells and NK cells. Expression appears to be downregulated upon differentiation of naive CD4+ T cells (Chen et al., 2000). WSX-1/TCCR-deficient mice were recently reported to have impaired Th1 responses when challenged in vivo with either protein antigen or the intracellular pathogen *Listeria monocytogenes* as measured by the production of IFN-γ. Moreover, these mice displayed markedly reduced levels of antigen-specific IgG2a antibodies, which are dependent on Th1 cells. IL-12 signaling in these mice was normal. These findings strongly support our identification of IL-27 as a ligand for WSX-1/TCCR with biologic activity on naive CD4+ T cells leading to proliferation and production of IFN-γ. In a second study using receptor-deficient mice, WSX-1/TCCR was found to be essential for the initiation phase of Th1 responses but was dispensable for their maintenance (Yoshida et al., 2001). This second study, combined with the notion that WSX-1/TCCR expression is downregulated upon polarization of naive T cells to fully committed Th1 cells, strongly

supports our finding that IL-27 is an early product of pathogen- or LPS-stimulated APCs and elicits a potent response on naive T cells independently of IL-12. We do not yet know if IL-27, in fact, might be required to sensitize naive T cells to IL-12 or whether the initial IFN-γ production by IL-27 responsive naive T cells and NK cells is necessary to enhance the production of IL-12 by APCs. Interestingly, the role of IL-12 as a key determinant of Th1 subset selection was recently challenged when it was found that an effective in vivo Th1 response mediating host resistance to infection can be induced in the absence of IL-12 (and IL-23) (Jankovic et al., 2002). Based on our findings, it is likely that the IFN-γ^+ $CD4^+$ T cells and IFN-γ producing NK cells that emerge in response to intracellular pathogens in IL-12-deficient animals are the result of the action of IL-27 functioning independently of IL-12. Studies are in progress.

The EBI3 subunit of IL-27 was originally identified as a homolog of IL-12 p40, expressed by EBV-transformed B cells (Devergne et al., 1996). EBI3 has been reported to associate with the p35 subunit of IL-12, but the functional significance of this interaction is not clear (Devergne et al., 1997). In vivo, EBI3 is expressed in spleen and tonsils and at very high levels in term placenta. Immunohistochemical analysis has revealed that EBI3 is expressed throughout pregnancy only by differentiated fetal trophoblasts that are in direct contact with maternal blood and maternal uterine NK cells (Devergne et al., 2001). Very high levels of EBI3 were found in peripheral blood from pregnant women, reaching levels of up to 400 ng/ml at full-term normal pregnancies. Only EBI3 was detected but not, e.g., IL-12p40. A dimeric form of the IL-12p40 subunit has been reported to antagonize IL-12 by competing for the IL-12R (Ling et al., 1995). Likewise, EBI3 might compete for the IL-27R, thereby dampening a systemic inflammatory response during normal pregnancy. Similarly, the excess latent EBI3 produced by APCs might function to prevent any further IL-27 stimulation of naive T cells after the initial response.

The discovery of IL-27 extends the number of factors that drive naive T cells to committed memory Th1 cells to three. Whereas our finding that IL-27 promotes vigorous growth of activated naive T cells suggests that IL-27 plays a role in the rapid initiation of a response to an inflammatory challenge, it appears to be unnecessary for its maintenance. IL-12, on the other hand, plays an important role in both primary and recall responses but requires upregulation of IL-12Rβ2 on naive T cells. Finally, IL-23 does not affect naive T cells. Instead it acts on the memory T cell compartment. We propose that these three factors act sequentially and at different stages of T cell costimulation. Although some functional overlap may exist, in particular between IL-27 and IL-12 as well as IL-12 and IL-23, indications are that these three cytokines are not redundant but instead have unique functions at specific times during the development of a pathogen-induced immune response. Contributions of all three factors might be critical in successfully resolving an immunological challenge. This fine-tuning of the response might offer new therapeutic opportunities for intervention at different stages of Th1-mediated pathologies.

Experimental Procedures

Identification of Human and Mouse IL-27

A structural alignment of available IL-6 family cytokine folds from FSSP (Holm and Sander, 1998) was profile aligned to other sequences (including distant species variants, GPA and viral IL-6's) with ClustalX (Thompson et al., 1997) with some manual adjustment. A weighted profile (Thompson et al., 1994) of the most conserved region of the fold, the C-terminal D-helix segment, was created. Fast scans of sequence databases on a Bioccelerator machine (Compugen, Tel Aviv, Israel) with the Profilesearch program (Gribskov et al., 1987) identified human EST AI085007 and mouse EST AA266872 which—in combination with genomic sequence—were used for the computational reconstruction of the human and mouse p28 sequences and the cloning of the full-length cDNAs.

Construction of Transient Expression Vectors

For P28, cDNAs encoding full-length human and mouse p28 were cloned into the pCDM8-etag vector via HindIII/XhoI (h/mp28-E). For EBI3, human and mouse EBI3 were cloned into pME18S-Ig vector via EcoRI/XhoI (h/mEBI3-Ig) and the mature portion of human EBI3 into pFlagCMV-1 vector via HindIII/NotI (F-hEBI3). For one-chain fusions EBI3/p28, HindIII/XbaI fragments were generated encoding the mature part of human or mouse EBI3, followed by the synthetic linker GSGSGGSGGSGSGKL and by the mature coding sequence of human or mouse p28 via HindIII/NotI. Fragments were inserted into pFLAG-CMV-1 using HindIII/NotI sites. For WSX-1, the preprotrypsin leader peptide and the flagtag encoding part of pFlagCMV-1 vector were deleted by PCR, instead a C-terminal $RGSH_6$-tag was introduced via SalI/SmaI (pCMV-1-$RGSH_6$); the cDNA encoding the extracellular part of human WSX-1 was cloned into this vector via HindIII/SalI (soluble hWSX-1-R). Proteins were produced via transient expression in HEK293T cells. For experiments requiring pure proteins, purification was performed by affinity chromatography.

Transient Transfection, Metabolic Labeling, and Immunoprecipitation

10^6 HEK293T cells were transiently transfected, cultured, and metabolically labeled as described (Oppmann et al., 2000). Proteins were precipitated from supernatants or cell lysates with either anti-Flag M2 agarose (Sigma), with anti-etag mAb bound to protein G Sepharose (Amersham Pharmacia), or with protein G Sepharose only.

Retroviral Constructs

The mature part of human and mouse WSX-1 was cloned into pMX vector via HindIII/NotI, and then a sequence encoding the preprotrypsin leader peptide fused to a flag epitope was cloned into the vector in frame and 5′ of WSX-1 via BamHI/HindIII (F-h/mWSX-1). Retrovirus obtained by transfection of BOSC23 cells was used to infect parental Ba/F3 cells, and cell surface expression of the desired proteins was monitored using a flag-PE-staining in FACS.

Cell Surface Ligand Binding Assay

Parental Ba/F3 cells and Ba/F3 cells expressing human or mouse F-WSX-1 were incubated with the supernatants from transiently transfected HEK293T cells containing human or mouse p28-E and/or EBI3-Ig for 2 hr. Binding of p28-E and EBI3-Ig to the cell surface was monitored by FACS using tag-specific antibodies. Control stains were performed using isotype control primary antibodies.

Coimmunoprecipitation Experiments

Proteins from supernatants of transiently transfected HEK293T cells containing F-hEBI3 or coexpressed hp28-E/F-hEBI3 were pulled down using either Flag M2-agarose, protein G Sepharose-coupled anti-etag mAb, or protein G Sepharose-coupled anti-H_5 mAb. The primary pricipitates were washed and then incubated with HEK293T cell supernatants containing shWSX-1-R. Secondary precipitates were separated by SDS-PAGE and subjected to Western blot. Precipitated proteins were visualized by ECL using antibodies against the respective protein tags.

Quantitation of mRNA Expression

cDNAs from various libraries or cultured macrophages and dendritic cells were prepared as described (Bolin et al., 1997) and used as templates for quantitative PCR. Fifty nanograms cDNA was analyzed for expression of human and mouse p28 and EBI3 by the fluorogenic 5′-nuclease PCR assay (Holland et al., 1991) using the ABI Prism 7700 Sequence Detection System (Perkin-Elmer). Analysis of cDNA samples was corrected for expression of 18S rRNA using a VIC-labeled probe (Perkin-Elmer, Foster City, CA) in multiplex reactions. The following primers were used. hEBI-3: f, CCGAGCCAGGTACTA CGTCC; r, CCAGTCACTCAGTTCCCCGT; probe, 6FAM-TGGCGGC TCAGGACCTCACAGA-TAMRA. mEBI3: f, ACCCATTGAAGCCACGA CTT; r, AGTATTGCATCCAGGTGTCAGCT; probe, 6FAM-TCACCC TCAGGAACTCGAAACCCCA-TAMRA. hp28: f, GCGGAATCTCACC TGCCA; r, GGAAACATCAGGGAGCTGCTC; probe, 6FAM-AGTGAA CCTGTACCTCCTGCCCCTGG-TAMRA. mp28: f, GGCCATGAGGC TGGATCTC; r, AACATTTGAATCCTGCAGCCA; probe, 6FAM-TGCA CAGGCACCTCCGCTTTCAG-TAMRA. Primers for hIL-12p35 and hIL-12p40 were obtained form Perkin Elmer as predeveloped assay reagents.

Mouse T Cell Proliferation Assay and IFN-γ Production

$CD4^+CD45RB^{high}$ or $CD4^+CD45RB^{low}$ T cell subsets were purified from the spleen and mesenteric lymph nodes of >6 month-old $IL\text{-}10^{-/-}$ C57/B6 N12 mice as described (Davidson et al., 1998). Cells were fractionated into $CD4^+CD45RB^{high}$ and $CD4^+CD45RB^{low}$ cell populations by two-color sorting on a FACSVantage SE (BD). The populations were >99% pure upon reanalysis. $CD4^+CD45RB^{high}$ or $CD4^+CD45RB^{low}$ were put into a proliferation assay with plate-bound anti-CD3 (145.2C11, in house) stimulation as described (Davidson et al., 1998). Additions to the growth media were anti-IL-2 mAb (JES6-1A12, in house, 10 μg/ml), anti-CD28 (in house,10 μg/ml), and cytokines as indicated. Cells were incubated for 5 days (37°C, 5% CO_2) with [^{3}H]TdR (Amersham) added at a final concentration of 1 μCi/well for the last 24 hr of incubation.

Sorted mouse $CD4^+CD45RB^{high}$ T cells were cultured with plate-bound anti-CD3 mAb. IL-27 (50 μg/ml) and IL-12 (50 μg/ml) were added in the absence or presence of anti-CD28 mAb (10 μg/ml). After 4 days, IFN-γ production induced by the respective factors was quantified by ELISA.

IL-13 Production Assay on Mouse T Cells

Sorted mouse $CD4^+CD45RB^{high}$ T cells were cultured with plate-bound anti-CD3 mAb, anti-CD28 mAb (10 μg/ml), IL-27 (10 μg/ml), and/or IL-4 (10 μg/ml) as indicated. After 4 days, IL-13 expression induced by the respective factors was quantified by ELISA (R&D).

Human CD45RA/CD45RO T Cell Proliferation and IFN-γ Production

FACS-purified CD45RA and CD45RO T cells (purity > 99%) were cultured at a density of 4×10^4 cells/well in a 96-well plate coated with anti-CD3 antibody (WT-31, in house, 10 μg/ml) and soluble anti-CD28 (BD, 1 μg/ml) with or without IL-27. Anti-hIL-2 mAb (17H12) and anti-hIL-2R mAb (B-B10), both from Diaclone, were added (10 μg/ml each). Cells were incubated for 5 days and then pulse labeled with [^{3}H]TdR (Amersham) for 8 hr.

Sorted human $CD4^+CD45RA$ T cells cultured with plate-bound anti-CD3 mAb. IL-27 (100 ng/ml) and IL-12 (2 ng/ml) were added in the absence or presence of anti-CD28 mAb (1 μg/ml). After 4 days, IFN-γ production induced was quantified by ELISA (Pharmingen).

Isolation and Culture of Human Monocytes

Monocytes were isolated from human peripheral blood as described (Sallusto and Lanzavecchia, 1994; Grouard et al., 1997). Total PBMCs were centrifuged on 52% Percoll (Amersham Pharmacia Biotech). The low-density cells were stained, and $CD14^+CD11c^+$ cells were isolated by cell sorting, purity >98%. Monocytes were cultured for 6 days in RPMI 1640, 10% FCS, 2 mM L-glutamine, 10mM HEPES, and 1mM sodium pyruvate/penicillin G/streptomycin in the presence of 100 ng/ml GM-CSF and 200 U/ml IL-4 (both Schering-Plough). The resulting monocyte-derived immature DCs were washed and cultured with 1 μg/ml LPS from *E. coli* (Sigma) at $3\text{–}5 \times 10^5$ cells/ml. Alternatively, DCs were cocultured with irradiated (7000 rad) L cells expressing CD40 ligand in the absence or presence of LPS (1 μg/ml) and IFN-γ (100 U/ml).

Detection of hIL-27 by Sandwich-ELISA

A pair of mAbs (19E5 and 7E5, both at 2.5 μg/ml) obtained from immunizing rats with recombinant hIL-27 was used for a sandwich-ELISA to detect hIL-27. 19E5 was used for coating; 7E5 was biotinylated and used for detection. Standard curves using purified recombinant hIL-27 or hEBI3 were generated. Cellular supernatants from DCs were harvested after 36 hr of stimulation, and 4-fold concentrated samples of 0.1 ml were loaded.

Isolation and Culture of Human NK Cells

NK cells were isolated from human PBMC by removal of adherent cells, positive selection on CD56 MicroBeads (Miltenyi, Auburn, CA), and FACS sorting of the $CD56^+CD3^-$ cells, resulting in >99% purity. NK cells were cultured at a density of 1×10^5 cells/well in Yssel's medium supplemented with 1% human AB serum in the presence of IL-2 (100 U/ml, R&D), IL-12 (2 ng/ml, R&D), and/or IL-27 (100 ng/ml). Culture supernatants were harvested after 72 hr, and IFN-γ production was determined by ELISA (Pharmingen).

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